Plant Physiol. (1985) 79, 24–27
0032-0889/85/79/0024/04/$01.00/0

Relationship between Stress-Induced ABA and Proline Accumulations and ABA-Induced Proline Accumulation in Excised Barley Leaves[1]

Received for publication February 25, 1985 and in revised form May 1, 1985

CECIL R. STEWART* AND GARY VOETBERG
Department of Botany, Iowa State University, Ames, Iowa 50011

ABSTRACT

When excised second leaves from 2-week-old barley (*Hordeum vulgare* var Larker) plants were incubated in a wilted condition, abscisic acid (ABA) levels increased to 0.6 nanomole per gram fresh weight at 4 hours then declined to about 0.3 nanomole per gram fresh weight and remained at that level until rehydrated. Proline levels began to increase at about 4 hours and continued to increase as long as the ABA levels were 0.3 nanomole per gram fresh weight or greater. Upon rehydration, proline levels declined when the ABA levels fell below 0.3 nanomole per gram fresh weight.

Proline accumulation was induced in turgid barley leaves by ABA addition. When the amount of ABA added to leaves was varied, it was observed that a level of 0.3 nanomole ABA per gram fresh weight for a period of about 2 hours was required before proline accumulation was induced. However, the rate of proline accumulation was slower in ABA-treated leaves than in wilted leaves at comparable ABA levels. Thus, the threshold level of ABA for proline accumulation appeared to be similar for wilted leaves where ABA increased endogenously and for turgid leaves where ABA was added exogenously. However, the rate of proline accumulation was more dependent on ABA levels in turgid leaves to which ABA was added exogenously than in wilted leaves.

Salt-induced proline accumulation was not preceded by increases in ABA levels comparable to those observed in wilted leaves. Levels of less than 0.2 nanomole ABA per gram fresh weight were measured 1 hour after exposure to salt and they declined rapidly to the control level by 3 hours. Proline accumulation commenced at about 9 hours. Thus, ABA accumulation did not appear to be involved in salt-induced proline accumulation.

Both ABA and proline accumulate in response to drought stress in a number of plants (3), and in barley, the two compounds have been measured in the same experiments (2). Proline accumulates in response to salt stress in a number of plants and barley is a well-studied example (3, 5, 15, 16). Application of ABA induces proline accumulation in *Hordeum* and *Lolium* leaves (4, 12). The lack of proline accumulation in response to ABA treatment has been reported for spinach, *Pennisetum thyphoides*, (10) tobacco, and sunflower leaves (3) and after several attempts we have not observed ABA-induced proline accumulation in bean leaves (C. R. Stewart and G. Voetberg, unpublished results). The metabolic processes leading to proline accumulation under all these treatments are similar (5, 11, 12). They include increased proline synthesis and inhibition of proline utilization by both oxidation and protein synthesis.

In pursuing the goal of understanding metabolic and cellular phenomena that lead to stress-induced proline accumulation, we have been interested in determining the relation, if any, between proline and ABA accumulations. Is the accumulation of ABA required for proline accumulation and can we identify other requisite processes such as the suggested (17) subcellular redistribution of solutes? The fact that ABA does not induce proline accumulation in several species which do accumulate proline under stress, could be used to argue that ABA accumulation is not involved in stress-induced proline accumulation. Because of problems of penetration and metabolism of applied ABA, it seems to us that the failure to induce proline accumulation by applied ABA in some plants is not sufficient to eliminate it from having a role in stress-induced responses. Differences among species in the time courses and amounts of stress-induced ABA accumulation have been reported (7). These differences might be partially related to differences in rates of metabolism. On the other hand, if ABA is shown to play a role in proline accumulation in one or more plants, the failure of ABA to cause it in some plants must be explained. Of course it is possible that ABA plays a role in wilting-induced proline accumulation only in a limited number of species (10).

In relatively long-term experiments with intact barley plants, ABA accumulation preceded proline accumulation and, when the plants were rehydrated, a decline in ABA preceded the disappearance of proline (2). This observation is consistent with, but does not demonstrate, a role for ABA in causing stress-induced proline accumulation. This paper reports more detailed time-course measurements along with some dose-response experiments designed to further describe the relation between ABA and proline accumulation in stressed barley. We have limited these measurements to wilting, ABA, and salt stress because the metabolic effects leading to proline accumulation have been determined for these treatments.

MATERIALS AND METHODS

Samples for proline and ABA measurements consisted of fully expanded second leaves excised from 2-week-old barley (*Hordeum vulgare* L., var Larker) plants grown as previously described (12). Treatments were as previously described for wilting (12), ABA addition (12), and salt shock (5). All additions and incubation solutions contained 50 mM sucrose and 1 mM glutamate and the leaves used in the wilting experiments were pretreated with sucrose and glutamate for at least 12 h to ensure an adequate supply of precursors for proline accumulation. No changes in ABA or proline occurred during the preincubation. No bacterial contamination of the sucrose and glutamate solution was appar-

[1] Supported by Department of Energy Contract No. DE-AC02-82ER12028.

ent in any of the experiments reported. Single-leaf samples (three replicates) were extracted and analyzed for proline as before (13).

Samples for ABA determination consisted of 50 leaves (10 g). Leaves were frozen in liquid N_2, stored at −20°C for no longer than 10 d, then freeze dried. Freeze-dried tissue (1 g) was homogenized in 20 ml of methanol-ethyl acetate-acetic acid, 50:50:1 (v/v/v), containing 20 mg/L butylated hydroxytoluene as an antioxidant, filtered through Whatman No. 1 filter paper, then made to a volume of 100 ml (8). Tritiated ABA (1.44 TBq/mmol, Amersham) was added during grinding as an internal standard. Filtrates were evaporated to dryness *in vacuo* at 35°C. Residue was extracted with 10 ml 500 mM K-phosphate (pH 8), filtered, and then partitioned 3 times (10 ml) into ethyl acetate by lowering the pH to 2.5 (8). Ethyl acetate was evaporated under an air stream.

Samples were dissolved in 5 ml of methylene chloride and filtered through Millipore prefilters. Filtrates were evaporated under air. Samples were redissolved in 100 μl 1% (v/v) acetic acid in methanol and 80 μl injected onto a preparative C_{18} (25 cm × 10 mm i.d.) HPLC column (1). Isocratic elution was with 40:60:1 (v/v/v) methanol:water:acetic acid at 2.5 ml/min (9). Fractions with retention times (29 to 31 min) corresponding to standard ABA (A_{254}) were pooled and dried under air. These fractions were redissolved in 20 μl 1% (v/v) acetic acid in methanol and 10 μl injected onto an analytical C_{18} (15 cm × 4.6 mm i.d.) HPLC column (1). Isocratic elution was with acetonitrile:water:formic acid (25:74.9:0.1, v/v/v) at 1 ml/min. Fractions with retention times (8–9 min) corresponding to standard ABA (A_{254}) were collected and radioactivity determined. Quantification was by comparison of sample A_{254} with that of ABA standards. Isotope dilution measurements were used to correct for losses during the procedures.

The sample size used contained sufficient ABA in control samples for accurate quantitation (>0.1 nmol). Much greater quantities were present in the wilted and ABA-treated samples. The ABA peak was adequately separated from adjacent peaks to allow for quantititative determination. Generally, about 40% of the ABA was recovered, the highest being 60%. Methyl ester of the ABA recovered from HPLC was separated by GC and identified with a mass selective detector.

RESULTS

Effects of Wilting. When excised second leaves were wilted, then incubated in that condition for a period of 24 h, the ABA level increased rapidly from a control level of about 0.1 to over 0.6 nmol/g fresh weight in a period of 4 h (Fig. 1A), then declined to about 0.3 nmol/g fresh weight. This level was maintained to 24 h. Upon rehydration, the ABA level rapidly declined to near-control levels in 4 h. The ABA levels in excised leaves incubated in a turgid condition (control leaves) increased only slightly over the 48-h incubation period compared to the wilted leaves. More detailed measurements of ABA levels at shorter time intervals in turgid leaves in similar experiments not shown here revealed no increases in ABA above the control levels reported in Figures 1 and 2. Proline levels in the wilted leaves began to increase after a 4-h lag and continued to increase throughout the 24-h period. Upon rehydration at 24 h, the proline levels declined but at a slower relative rate than the ABA. The proline levels began to decline immediately as the ABA levels fell below the apparent threshold value of approximately 0.3 nmol/g fresh weight.

When wilted leaves were rehydrated 8 h after wilting, the ABA levels again rapidly declined (Fig. 1B). Proline also declined rapidly upon rehydration as the ABA levels fell below a value of 0.3 to 0.4 nmol/g fresh weight.

Leaves wilted and incubated in a wilted condition for less than 8 h before rehydration are shown in Figure 2. Three h was a sufficient time for ABA levels to reach the 0.6 nmol/g fresh

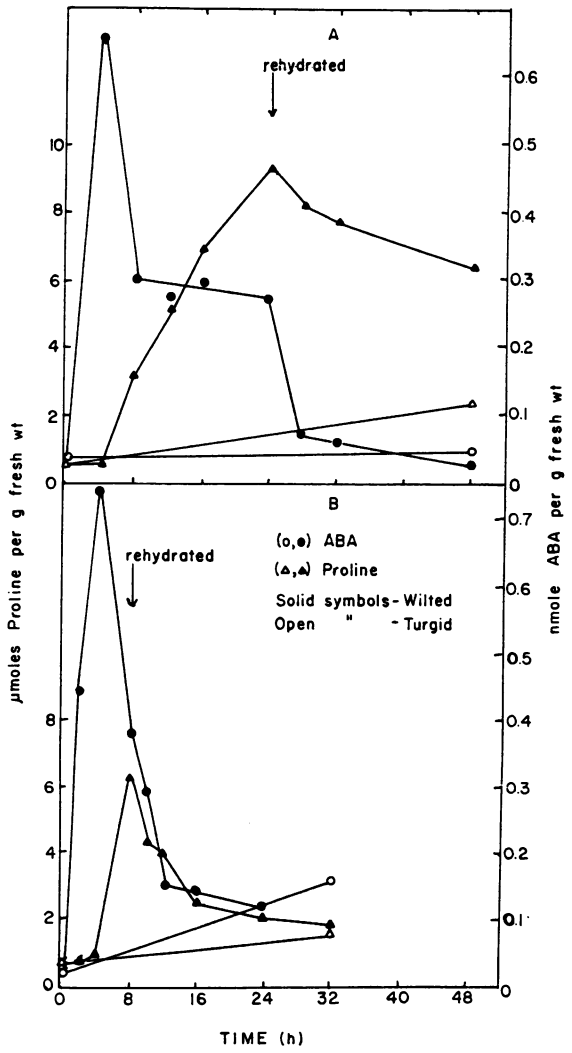

FIG. 1. Time course of ABA (○, ●) and proline (Δ, ▲) levels in wilted and turgid, excised second leaves of barley. Leaves were pretreated with the cut end in a solution of 50 mM sucrose and 1 mM glutamate for 12 h prior to wilting; 25% of the fresh weight was lost during a 1-h wilt period starting at zero time. Leaves were rehydrated by placing the cut end in sucrose and glutamate. (A), Rehydrated after 24 h; (B), rehydrated after 8 h.

weight value and, when rehydrated at 3 h, these levels declined rapidly, falling below 0.2 nmol/g fresh weight by the 5-h sampling time (Fig. 2A). Proline accumulation commenced between 3 and 5 h and continued for 2 or more h. Only slight increases were observed after 5 h when the ABA levels were less than 0.3 nmol/g fresh weight. No decline in the proline levels was observed in this experiment. Such levels never exceeded 2 μmol/g fresh weight. Incubation of leaves in a wilted condition for only 1 h was sufficient to induce ABA accumulation; the level reached 0.1 nmol/g fresh weight and continued to increase an additional h after rehydration (Fig. 2B). The ABA levels never reached the 0.3 nmol/g fresh weight value in this experiment and proline accumulation was not induced in these leaves.

Effects of Exogenous ABA. Variable doses of ABA were applied to excised barley leaves by allowing leaves to take up different concentrations of ABA solutions through the cut end for variable time periods. When turgid leaves were allowed to take up a 10 mg/L solution for 20 min, then removed from the ABA solution, the ABA level reached 1.6 nmol/g fresh weight (Fig. 3A). Subsequently, the ABA levels declined to 0.8 ± 0.2

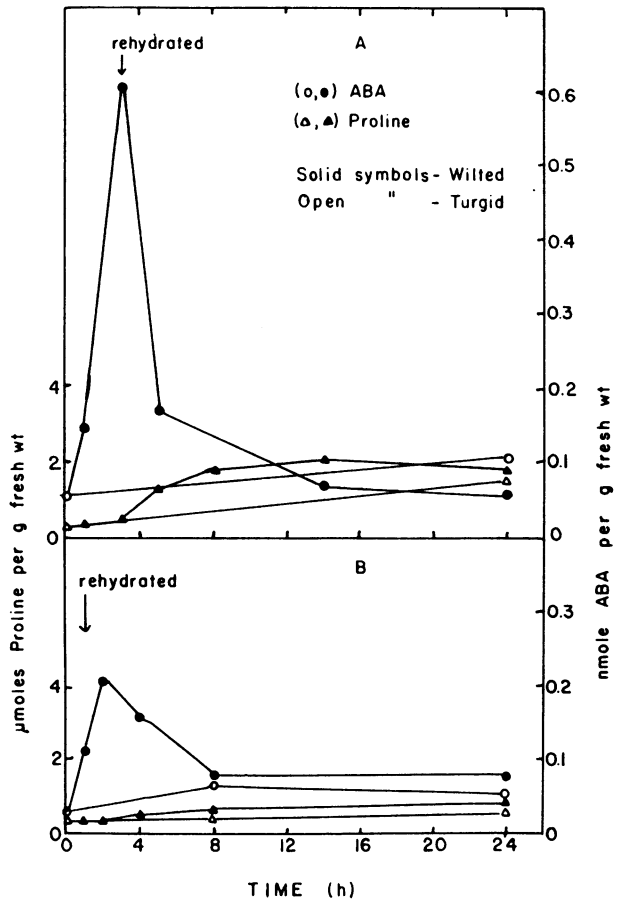

FIG. 2. Time course of ABA (O, ●) and proline (Δ, ▲) levels in wilted and turgid excised barley leaves. Experimental details described in Figure 1. (A), Rehydrated after 3 h; (B), rehydrated after 1 h.

nmol/g fresh weight by 4 h and remained within this range to the end of the experiment (24 h). Proline accumulation began 2 to 4 h after ABA addition and continued to the end of the experiment.

The addition of less ABA (10 min in a 5 mg/L solution) resulted in leaf ABA levels of about 0.4 nmol/g fresh weight 10 min later (Fig. 3B). ABA levels gradually declined during the following 24-h period. They fell below the 0.3 nmol/g fresh weight by about 2 h. Proline accumulation was induced by this treatment and it began to accumulate after 4 h and continued to do so at a slow rate until the end of the experiment. Adding 2 mg/L ABA to leaves for 10 min resulted in ABA levels of about 0.4 nmol/g fresh weight but only for a short time because the level had declined to nearly the control level by 4 h after its addition (Fig. 3C). Proline accumulation was not induced in these leaves by this treatment.

Effects of Salt. The ABA and proline content of salt-shocked leaves is shown in Figure 4. Similar to our previous report, proline was induced to accumulate after about 8 to 12 h, and it continued to accumulate over a 26-h period. Our previous work showed that proline levels would continue to increase and would not decline even if the leaves are removed from the salt. There was a relatively small increase (<0.2 compared to >0.6 nmol/g fresh weight in wilted leaves) increase in ABA measured after 0.5 h in salt, but all measurements after that showed ABA levels in salt-shocked leaves were not different from those in the controls.

DISCUSSION

The time course of ABA accumulation observed in these experiments shows a rapid increase in ABA followed by a decline

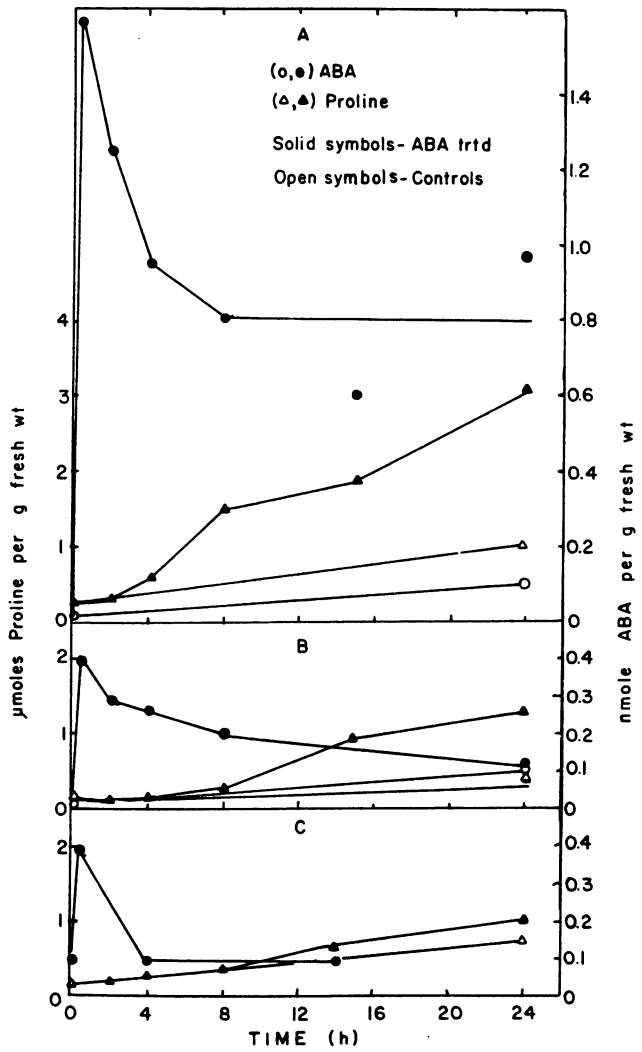

FIG. 3. Time course of ABA (O, ●) and proline (Δ, ▲) levels in turgid excised barley leaves treated with and without ABA. Prior to ABA treatment, leaves were incubated with the cut end in a solution of 50 mM sucrose and 1 mM glutamate for 12 h. ABA in the sucrose and glutamate solution was applied through the cut end starting at zero time. After removal from ABA-containing solution, leaves were incubated in the sucrose and glutamate solution. (A), Leaves in 10 mg/L ABA for 20 min; (B), leaves in 5 mg/L ABA for 10 min; (C), leaves in 2 mg/L ABA for 10 min.

to a steady level. This time course has been observed previously for pearl millet and rice (7) but generally ABA levels rise rapidly then level off after 3 to 5 h. The important point here is that ABA levels increased prior to proline and it appears that proline only accumulated while ABA levels were maintained above some threshold level (0.3 nmol/g fresh weight in this set of measurements). The decline in ABA and proline levels upon rehydration has been previously reported (2). Measurements at the time intervals used in these experiments show that the decline in ABA preceded the decline in proline content.

Added ABA induced proline to accumulate only when ABA levels exceeded a threshold level for a period of at least 2 h and, in these measurements, that level was similar to the threshold level in wilted leaves. ABA-induced proline accumulation appeared to be proportional to the ABA content.

Measurements recorded in this paper indicate correlations between ABA and proline accumulations that suggest they might be linked. We have not been able to measure proline accumula-

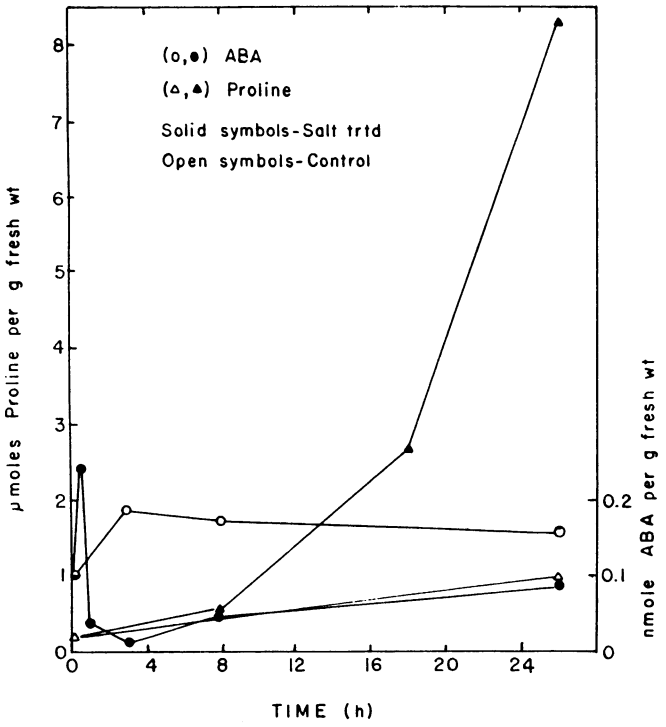

FIG. 4. Time course of ABA (O, ●) and proline (Δ, ▲) levels in salt-shocked and control excised barley leaves. Both NaCl (205 mM) and control solutions contained sucrose and glutamate.

tion in wilted barley leaves in the absence of preceding ABA accumulation. Added ABA induced proline accumulation at similar levels but at a slower rate. Rates of proline accumulation similar to rates induced by wilting are induced by higher concentrations of ABA (12).

Proline accumulation was induced in salt-shocked barley leaves without a preceding ABA accumulation to a similar threshold level and duration that was observed in wilted and ABA-treated leaves. It has been shown that turgor loss is necessary for ABA accumulation (6) and turgor is maintained in these salt treatments (5). Thus, the mechanisms that initiate proline accumulation in wilted and salt-shocked leaves could be different. Alternatively, a sequence of events could be initiated by wilting involving a turgor loss-induced ABA accumulation followed by another cellular process that would initiate proline accumulation. Salt might then initiate the second process in the absence of turgor loss-induced ABA accumulation. Subcellular compartmentation of inorganic ions has been suggested (17) to occur when proline accumulates and would be a candidate for this second cellular process.

If there is a cause and effect link between ABA and proline accumulation in barley, then why does ABA fail to induce proline accumulation in other species? One obvious possibility is that the link is present in some species and not in others. This difference could be related to differences in the metabolic mechanisms leading to proline accumulation. Alternatively, it may be that levels of ABA required to induce proline accumulation have not been achieved in other plants. These levels may be higher and the rate at which ABA is metabolized differs among species (7).

LITERATURE CITED

1. ACKERSON RC 1980 Stomatal response of cotton to water stress and abscisic acid as affected by water stress history. Plant Physiol 65: 455–459
2. ASPINALL D 1980 Role of abscisic acid and other hormones in adaptation to water stress. *In* NC Turner, PJ Kramer, eds, Adaptation of Plants to Water and High Temperature Stress. John Wiley and Sons, New York, p 155
3. ASPINALL D, LG PALEG 1981 Proline accumulation: physiological aspects. *In* LG Paleg, D Aspinall, eds, Physiology and Biochemistry of Drought Resistance in Plants. Academic Press, Sydney, p 205
4. ASPINALL D, TN SINGH, LG PALEG 1973 Stress Metabolism V. Abscisic acid and nitrogen metabolism in barley and *Lolium temulentum* L. Aust J Biol Sci 26: 319–327
5. BUHL MB, CR STEWART 1983 Effects of NaCl on proline synthesis and utilization in excised barley leaves. Plant Physiol 72: 664–667
6. CREELMAN RA, JAD ZEEVAART 1985 Abscisic acid accumulation in spinach leaf slices in the presence of penetrating and nonpenetrating solutes. Plant Physiol 77: 25–28
7. HENSON IE, SA QUARRIE 1981 Abscisic acid accumulation in detached cereal leaves in response to water stress I. Effects of incubation time and severity of stress. Z Pflanzenphysiol 101: 431–438
8. HUBICK KT, DM REID 1980 A rapid method for the extraction and analysis of abscisic acid from plant tissue. Plant Physiol 65: 523–525
9. MARKHART AH 1982 Penetration of soybean root systems by abscisic acid isomers. Plant Physiol 69: 1350–1352
10. MCDONNELL EM, SJ COUGHLAN, RG WYN JONES 1983 Differential effects of abscisic acid on glycinebetaine and proline accumulation in three plant species. Z Pflanzenphysiol 109: 207–213
11. STEWART CR 1981 Proline accumulation: biochemical aspects. *In* LG Paleg, D Aspinall, eds, Physiology and Biochemistry of Drought Resistance in Plants. Academic Press, Sydney, p 243
12. STEWART CR 1980 The mechanism of abscisic acid-induced proline accumulation in barley leaves. Plant Physiol 66: 230–233
13. STEWART CR, SF BOGGESS 1978 Metabolism of (5-^{3}H)proline by barley leaves and its use in measuring the effects of water stress on proline oxidation. Plant Physiol 61: 654–657
14. STEWART CR, SF BOGGESS, D ASPINALL, LG PALEG 1977 Inhibition of proline oxidation by water stress. Plant Physiol 59: 930–932
15. STEWART GR, JA LEE 1974 The role of proline accumulation in halophytes. Planta 120: 279–289
16. VOETBERG G, CR STEWART 1984 Steady state proline levels in salt-shocked barley leaves. Plant Physiol 76: 567–570
17. WYN JONES RG, R STOREY, RA LEIGH, N AHMAD, A POLLARD 1977 A hypothesis on cytoplasmic osmoregulation. *In* E Marre, O Cifferi, eds, Regulation of Cell Membrane Activities in Plants. North-Holland, New York, p 121